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Heterologous Protein Expression in the *Xenopus* Oocyte

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Abstract

The *Xenopus* oocyte is a specialized single cell of colossal size (>1 mm diameter) that is highly amenable for microinjection and a stalwart model for heterologous expression. Oocytes are easily obtainable, robust in vitro, and faithfully express injected constructs. Their large size translational capacity provides a huge canvas for observing and recording integrated cellular responses—from studies of single molecules within single cells to medium-throughput drug-screening applications. Most eukaryotic promoters suffice for *Xenopus* expression, and the oocyte can functionally express proteins from many diverse organisms. This protocol provides a basic introduction for scientists keen to perform nuclear microinjections of cDNA constructs. These are easy methods to master, do not require elaborate equipment, and make accessible a wonderful model cell system for studying signaling, transport, cell architecture, and protein function.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

cDNA (50–200 μg/mL)

Mineral oil (Sigma-Aldrich M5310)

Modified Barth's solution (MBS) <R>

> In my laboratory, we use MBS not only for oocyte incubation but also for oocyte preparation simply owing to paranoia over oocyte quality. Ringer's solution is an acceptable surrogate during routine oocyte preparation. Solutions may be supplemented with antibiotics other than gentamycin (for example, 1% penicillin/streptomycin).

Xenopus (adult females)

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Source adult female frogs from Xenopus Express (www.Xenopus.com), Xenopus 1 (www.Xenopus1.com), or Nasco (www.enasco.com). Guidance for maintaining a frog colony can be found elsewhere (Koustubhan et al. 2008; Delpire et al. 2011).

Oocytes pre-prepared for injection are also available (http://ecocyte-us.com); this can be a convenient option for infrequent or novice users. Oocytes from species other than Xenopus laevis (e.g., X. tropicalis or X. borealis) can be used. They are smaller and harder to inject compared with X. laevis oocytes, but advantages have been noted (Marchant and Parker 2001; Cristofori-Armstrong et al. 2015). Xenopus resource centers provide additional strains/lines (Pearl et al. 2012). Xenbase (www.xenbase.org) is an extensive portal collating Xenopus community resources.

Equipment

Borosilicate glass capillary tubes for making pulled pipettes (type dependent on microinjection equipment)

Dissection equipment

- Surgical scissors
- Watchmaker's forceps (Dumont #5 and #55)

Glass vials (20 mL; Research Products International 121001)

Incubator (16C–20°C)

Light box or gooseneck illuminator

Microinjection setup (see Step 7)

- Microinjection apparatus with nanoliter precision (with foot pedal, preferred)
- Micromanipulator (three-axis, preferred)

Micropipette puller (see Step 5)

Nutator (optional; see Step 3)

Oocyte holders for microinjection (see Step 8)

In my laboratory, we use customized plates (Petri dishes with secured nylon/polypropylene mesh [0.5–0.8 mm, Small Parts Inc.]).

Petri dishes

Stereomicroscopes

Separate stations for oocyte preparation and microinjection are needed.

METHOD

Bypass Steps 1 and 2 by purchasing prepared oocytes from commercial vendors.

1. Dissect ovarian lobes from donor frogs (see Protocol: **Isolation of *Xenopus* Oocytes** [Sive et al.2010]) and place in a Petri dish containing MBS.

2. Manually isolate at least 200 (or more, depending on the number/combination of constructs for injection) stage V–VI oocytes under a stereomicroscope by carefully "plucking" the larger (>1 mm diameter) oocytes from surrounding tissue while removing the enveloping follicular cell layers, which can impede clean penetration of the microinjection needle. Use two pairs of fine watchmaker's forceps to grasp the tissue connecting individual oocytes to the ovarian lobe, and gently move the forceps apart to tease back the follicular cell layers, like peeling skin from a grape.

   Each oocyte will deform and pop through the resulting opening as the enveloping cell layer is ruptured and removed by forceps passing around the cell circumference. This takes practice, and many cells will be damaged or burst. Periodically restart with a fresh lobe in a new Petri dish.

   This is the preferred method for oocyte isolation in my laboratory. Alternatively, bulk preparation of oocytes can be achieved by enzymatic defolliculation as described in Protocol: **Isolation of Xenopus Oocytes** (Newman et al. 2018).

   See Troubleshooting.

3. Separate the oocytes into 20-mL glass vials containing MBS (~50 oocytes per vial). Allow the oocytes (whether isolated manually or enzymatically) to recover in MBS overnight in an incubator at 16°C–20°C.

   If the oocytes were isolated enzymatically, perform overnight recovery on a nutator.

4. After overnight incubation, remove damaged oocytes. Discard cells with mottled pigment around the animal pole. Replace the MBS.

   Heathy oocytes retain a clearly demarked asymmetry in pigment between the animal (pigmented) and vegetal hemisphere.

   See Troubleshooting.

5. Prepare multiple needles for microinjection according to the instructions of the micropipette puller used.

   A sharp needle preserves oocyte viability, especially for nuclear microinjection. Needles should have a reasonable shank (up to 900 µm from the tip) and a tip of ~15 µm (outer diameter) after breakage under a microscope. A basic micropipette puller will meet these requirements.

6. Prepare the oocytes for microinjection. Under a stereomicroscope, align ~100 oocytes per construct within a nylon/polypropylene restraining mesh secured within a Petri dish containing MBS. Manipulate the oocytes to sit vegetal side down, with the animal (pigmented) pole upwards.

For cDNA injections, the oocyte nucleus (or germinal vesicle) must be injected. The germinal vesicle is large (40 nL in volume) and rests within the animal hemisphere. Oocytes must therefore be oriented animal pole up for microinjection.

For expression studies, oocytes can be injected with mRNA into the vegetal cytoplasm (see Protocol: **Microinjection of Xenopus Oocytes** [Aguero et al. 2018]) or with cDNA into the nucleus. Cytoplasmic injections are straightforward and well tolerated; injection is even possible before defolliculation (Maldifassi et al. 2016). Compared with cytoplasmic injection, germinal vesicle injection requires more time and results in poorer oocyte viability because of germinal vesicle damage, but cDNA constructs require less preparation and express through endogenous targeting pathways.

7. Load the needle on the microinjector with the solution containing cDNA (<3 ng of cDNA is injected into each oocyte) according to the instructions of the microinjector used.

Depending on the equipment used, this step commonly involves back-filling the needle with mineral oil, breaking the tip, and then front-filling with injection solution. Different microinjectors are available, ranging from handheld injectors (for mRNA injection), to widely used and recommended plunger-based systems (e.g., Drummond Nanoject II and III), and even automated injectors (Schnizler et al. 2003; Papke and Stokes 2010). Any stably mounted microinjector that reproducibly dispenses nanoliter volumes is suitable.

8. Align an oocyte under the injection needle, and manipulate the needle to the oocyte surface. Observe the oocyte surface dimple under pressure before penetration, and then watch as the needle disappears into the cell upon penetration. (At this point, the needle will be far enough into the cell nucleus for injection.) Inject once, pause, and then gently withdraw the needle. Move to the next oocyte and repeat. Periodically withdraw the tip of the needle from the solution to check that the needle remains unclogged.

The large volume of the oocyte nucleus (~40 nL) can tolerate a surprisingly large injection volume (<15 nL).We secure the Petri dish containing the oocytes on a moveable stage that can be ratcheted to position cells for injection. This maximizes throughput and minimizes risk of inadvertent needle damage.

See Troubleshooting.

9. After microinjection, place 20–30 oocytes into individual 20-mL glass vials containing 10–15 mL of MBS, and return to the 16°C–20°C incubator. Remove apoptotic oocytes daily to prevent deleterious effects on healthy cells.

See Troubleshooting.

10. Screen oocytes for expression of injected cDNA.

The method used for examining expression will depend on experimental goals and may involve imaging, electrophysiology, radioisotope flux, or western blotting. Oocytes can screened as early as 24 h after microinjection, although expression usually peaks over the following days.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 2): The quality of harvested oocytes is poor.

Solution: Oocyte quality is critical for success. If oocyte quality from multiple donor frogs is consistently poor, but better from freshly sourced animals, there is likely to be a husbandry problem (e.g., inappropriate water composition/pH, nutrition, or the presence of infectious agents) (Koustubhan et al. 2008; Delpire et al. 2011). If a husbandry issue is suspected, consult veterinary staff. Even under seemingly identical housing conditions, seasonal variations in oocyte quality occur. During these periods it may be simpler to directly source commercially prepared oocytes.

Problem (Step 4): The oocytes display poor viability in vitro.

Solution: Monitor the viability of uninjected oocytes in parallel with mock-injected oocytes to discriminate between problems with culture media/conditions and poor microinjection technique. If necessary, remake buffers with careful attention to buffer osmolarity/pH. If black spots appear on the oocyte surface, or the pigment shows excessive marbling, microbial contamination is likely (O'Connell et al. 2011). In this case, supplement with fresh antibiotics. Poor viability after isolation by enzymatic digestion can result from excessive collagenase exposure. This can be prevented by the manual defolliculation of oocytes, and although this procedure takes practice, oocyte quality is better.

Problem (Step 8): The needle does not dispense fluid.

Solution: Ensure that no air bubbles or clogged material became trapped within the needle during filling. The newer Nanoject III (Drummond) streamlines needle mounting procedures. If the needle is blocked, prepare a new needle.

Problem (Step 9): The cells die after microinjection.

Solution: A poorly fashioned microinjection needle or excessive injection volume will cause significant cell death the day after injection. When injecting, check for evidence of damage to the oocyte surface (a persistent white wound). If this occurs, then the needle is too broad or blunt, and new needles should be prepared. Contaminants in the injected material can cause cell death. Check the purity of injected material; plasmid DNA preparations can be purified with an endotoxin-free plasmid preparation kit or a PCR purification kit. Expressed proteins may also prove deleterious to oocyte viability.

Problem (Step 10): There is weak construct expression.

Solution: Low expression efficiency will result from a clogged microinjection needle. Any precipitates in the injected solution can be removed by centrifugation before backfilling. For cDNA injections, better visualization of the nucleus will help. This can be achieved by giving oocytes a brief pulse of centrifugation in a benchtop microcentrifuge to raise the nucleus toward the surface, or by using albino oocytes to visualize the nucleus under transillumination. Expression does vary from donor to donor (in any batch, 20%–80% of injected oocytes will express the injected construct) and from cell to cell (e.g., in observed fluorophore intensity or peak current magnitude).

RELATED INFORMATION

This protocol is an update to previously published procedures (see Protocol: **Nuclear Microinjection to Assess How Heterologously Expressed Proteins Impact Ca^{2+} Signals in *Xenopus* Oocytes** [Lin-Moshier and Marchant 2013a]; Protocol: **A Rapid Western Blotting Protocol for the *Xenopus* Oocyte** [Lin-Moshier and Marchant 2013b]; and Introduction: **The *Xenopus* Oocyte: A Single-Cell Model for Studying Ca^{2+} Signaling** [Lin-Moshier and Marchant 2013c]).

Further information on the isolation of *Xenopus* oocytes can be found in Sive et al. (2000) and in Protocol: **Isolation of *Xenopus* Oocytes** (Newman et al. 2018). Different laboratories use iterated versions of these core methods, which can be consulted in conjunction with cytoplasmic oocyte microinjection protocols (see Protocol: **Microinjection of *Xenopus* Oocytes** [Aguero et al. 2018]) and helpful video resources that are available online (Cohen et al. 2009; Maldifassi et al. 2016).

RECIPE

Modified Barth's Solution (MBS)

Reagent	Final concentration (1×)
NaCl	88 mM
KCl	1 mM
$NaHCO_3$	2.4 mM
$MgSO_4 \cdot 7H_2O$	0.82 mM
$Ca(NO_3)_2 \cdot 4H_2O$	0.33 mM
$CaCl_2 \cdot 2H_2O$	0.41 mM
HEPES	5 mM

Prepare a stock solution (5×, pH 7.4), which can be stored for several months at −20°C. Prepare working solutions by dilution, and autoclave prior to use. After autoclaving, add gentamycin (final concentration in 1× solution, 50 μg/mL).

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